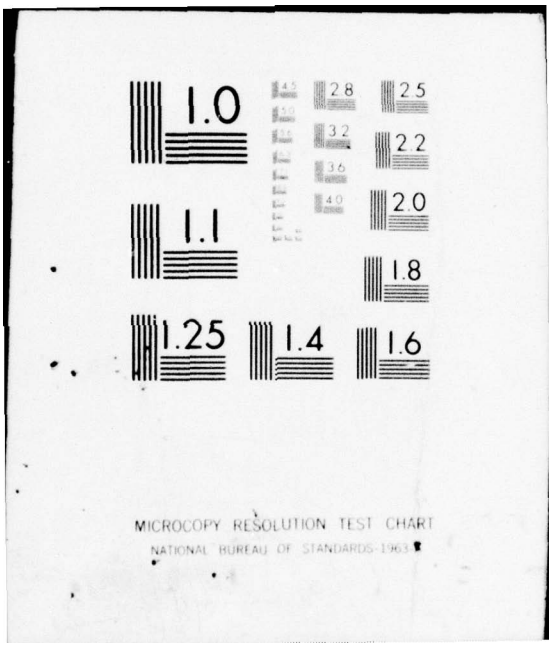
AD-A033 232 ARMED FORCES RADIOBIOLOGY RESEARCH INST BETHESDA MD F/G 6/5
LOCALIZATION OF 99M SUB T C DIPHOSPHONATE IN ACUTELY INJURED MU--ETC(U)
AUG 76 B A SIEGEL, W K ENGEL, E C DERRER

UNCLASSIFIED AFRRI-SR76-45 NL

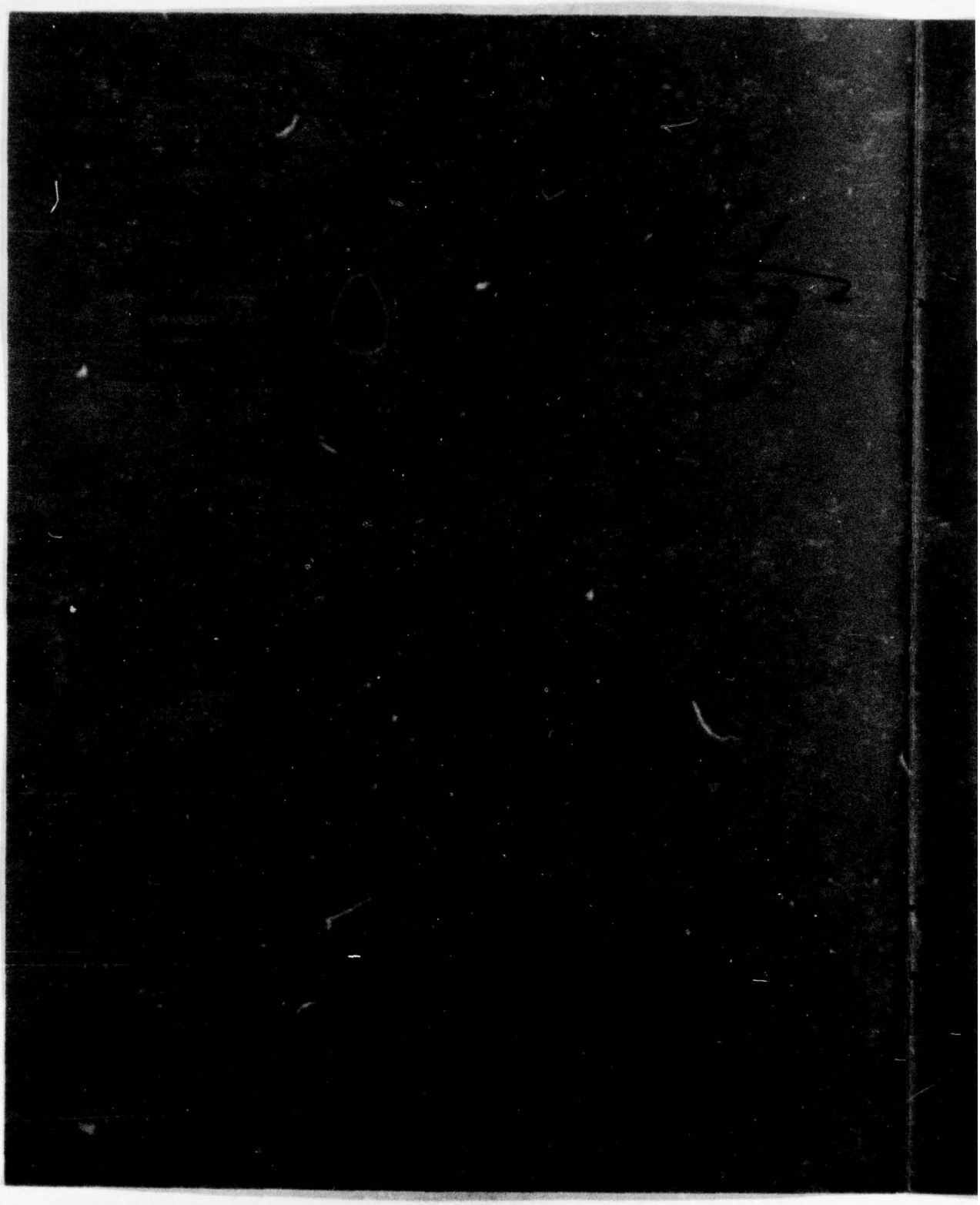
1 OF 1
AD
A033232

END

DATE
FILMED
2-77


1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

ADA033232



UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

sub / Tc

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER: AFRRI-SR76-45

2. GOVT ACCESSION NO.

3. RECIPIENT'S CATALOG NUMBER

(6) 4. TITLE (and Subtitle): LOCALIZATION OF ^{99m}Tc DIPHOSPHONATE IN ACUTELY INJURED MUSCLE: RELATIONSHIP TO MUSCLE CALCIUM DEPOSITION

5. TYPE OF REPORT & PERIOD COVERED: Scientific rept.

6. PERFORMING ORG. REPORT NUMBER

(10) 7. AUTHOR(s): B. A. Siegel, W. K. Engel* and E. C. Derrer*

(*National Institutes of Health)

8. CONTRACT OR GRANT NUMBER(s)

9. PERFORMING ORGANIZATION NAME AND ADDRESS: Armed Forces Radiobiology Research Institute (AFRRI), Defense Nuclear Agency, Bethesda, Maryland 20014

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: NWED QAXM, 0911 20

(12) 28p.

11. CONTROLLING OFFICE NAME AND ADDRESS: Director, Defense Nuclear Agency (DNA), Washington, D. C. 20305

(11) 12. REPORT DATE: August 1976

13. NUMBER OF PAGES: 29

(14) 14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office): AFRRI-SR76-45

15. SECURITY CLASS. (of this report): UNCLASSIFIED

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Tc(99m)

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

There is an operational requirement in military nuclear medicine to provide more effective methods for evaluating changes in physiological and organ function in trauma. In rats with experimental ischemic myopathy there was a significant correlation ($r = 0.778$, $p < 0.001$) between muscle uptake of ^{99m}Tc diphosphonate (EHDP) and tissue calcium concentration. In addition, the accumulation of both calcium and ^{99m}Tc EHDP in acutely injured muscles was further increased in rats →

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

034700

/B

20. ABSTRACT (continued)

with vitamin D-induced hypercalcemia. Histologic studies demonstrated staining of damaged muscle fibers with alizarin red, indicating the presence of microcrystalline or ultramicrocrystalline calcium salts. Staining of muscle fibers was most intense in the outer marginal zones of individual microscopic infarcts. Our results suggest that the uptake of ^{99m}Tc EHDP in acutely damaged skeletal muscle is directly related to the deposition of calcium salts within the injured muscle fibers. This work increases our understanding of the basic mechanisms of the pathogenesis of ^{99m}Tc diphosphonate localization in damaged muscle. The results can be applied toward improved clinical interpretations, and benefit patients (soldiers) with trauma, acutely injured muscle or other conditions which can be detected with these techniques.

PREFACE

We thank E. L. Barron, N. L. Fleming, M. E. Flynn, R. G. Hamilton, G. Hubbard, J. Jozsa, J. K. Warrenfeltz and G. C. Zirzow for technical assistance and Dr. A. Tofe of the Procter and Gamble Company for the generous supply of ^{3}H diphosphonate.

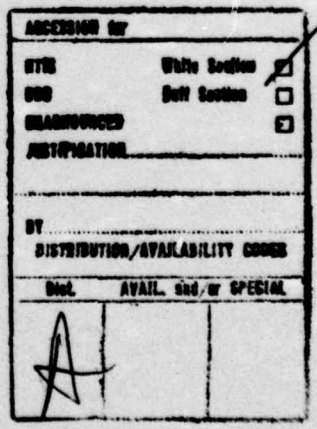


TABLE OF CONTENTS

	Page
Preface	1
Introduction	5
Methods	5
Animal model	5
Tracer distribution and tissue calcium studies	6
Plasma enzyme and calcium studies	7
Comparison study of ^{99m}Tc EHDP and ^{3}H EHDP	7
Histologic studies	7
Results	8
Tracer distribution and tissue calcium studies	8
Plasma enzyme and calcium studies	10
Comparison study of ^{99m}Tc EHDP and ^{3}H EHDP	11
Histologic studies	11
Discussion	16
References	21

PRECEDING PAGE BLANK-NOT FILMED

LIST OF FIGURES

Page

Figure 1. Relationship between muscle calcium concentration and ^{99m}Tc EHDP uptake 9

Figure 2. Relationship between muscle uptake of ^{3}H EHDP and ^{99m}Tc EHDP 12

Figure 3. Parallel sections from a large infarct 13

Figure 4. Modified trichrome stain of a normal region and an inner zone of a large infarct 14

Figure 5. Detail of the border between normal and infarct regions . . . 15

Figure 6. Detail of the inner zone of an infarct 15

LIST OF TABLES

Table 1. Muscle Diphosphonate, Calcium, and Potassium Concentrations 8

Table 2. Plasma Creatine Phosphokinase Concentrations 10

Table 3. Plasma Calcium Concentrations 11

Table 4. Summary of Histochemical Findings in Ischemic Myopathic Lesions 16

INTRODUCTION

It has recently been demonstrated that several ^{99m}Tc labeled radiopharmaceuticals localize in acutely damaged myocardium,[2,3,7,10,18,20,23,24,43,47,51] skeletal muscle,[20,45,50] and other tissues.[12,20] Clinical studies have shown the utility of these radiopharmaceuticals for imaging of recent myocardial infarcts[24,43] and active skeletal muscle inflammatory disease,[6,29] and several investigators have suggested that the intensity of the tissue tracer accumulation is closely linked to the extent and severity of the injury.[10,24,47,50,51]

The mechanism by which these compounds localize in damaged tissue is unknown. In the case of "bone-seeking" radiopharmaceuticals (^{99m}Tc labeled polyphosphate, pyrophosphate, and diphosphonate) it has been suggested that their localization is related to the deposition of calcium in injured cells.[2,7] Although there is abundant evidence that calcium accumulates in damaged myocardial and skeletal muscle cells[37,48,49,53] and that crystalline deposits resembling hydroxyapatite form in various organelles,[4,5,11,25,48,53] there is inconclusive evidence demonstrating the relation between calcium deposition and tracer accumulation.[8,32] To evaluate the pathogenesis of tracer localization in damaged muscle, in the present study we have correlated the tissue uptake of ^{99m}Tc-Sn-diphosphonate (ethane-1-hydroxy-1,1-diphosphonate (EHDP)) with the change in tissue calcium concentration in an animal model of acute skeletal muscle injury. In addition, vitamin D (as dihydrotachysterol) was administered to some experimental animals to test the hypothesis that hypercalcemia would result in increased deposition of calcium, and consequently, of ^{99m}Tc EHDP in damaged muscle. The histological localization of the calcium deposited in the injured muscle tissue has also been studied and correlated with other histochemical findings.

METHODS

Animal model. Male Osborne-Mendel rats weighing 140-180 g were used in all studies. Ischemic myopathy was induced in the manner previously

reported.[35,36,50] Briefly, under pentobarbital anesthesia, the abdominal aorta was ligated just proximal to its bifurcation and 5 days later, 5-hydroxytryptamine (5-HT) was administered intraperitoneally at a dosage of 15 mg/kg body weight. Animals undergoing ligature were fully recovered within 48 hours. In this model, only the hindlimb muscles are injured and the forelimb muscles can serve as control tissue in each animal. The induced injury is characterized by multiple microscopic infarcts, which have been considered histologically similar to the early lesions of Duchenne muscular dystrophy.[35] The damaged muscle also exhibits an acute decrease in potassium concentration and increased uptake of ^{99m}Tc EHDP.[50] Concomitantly, the animals have elevations of plasma creatine phosphokinase.[36]

To assess the effects of increased plasma calcium on the deposition of both calcium and ^{99m}Tc EHDP in injured muscle, some rats were pretreated with dihydrotachysterol (DHT). The drug (1 mg in 4 ml sesame oil) was administered by gastric tube 24 hours prior to 5-HT injection.

Tracer distribution and tissue calcium studies. The muscle uptake of ^{99m}Tc EHDP was determined in control rats (n = 5), animals treated only with DHT (n = 4), and ischemic myopathy rats with (n = 8) and without (n = 7) DHT pretreatment. Twenty-four hours after 5-HT injection and/or 48 hours after DHT administration, the rats were injected intravenously with ^{99m}Tc EHDP (0.1 mg, 300 μCi ^{99m}Tc, in 0.1 ml normal saline). Two hours later, 0.2- to 0.6-g samples of both quadriceps, both gastrocnemii, and both triceps brachialis were obtained, weighed, and counted in a NaI(Tl) crystal well counter along with dilute standards of the radiopharmaceutical. The uptake results were expressed as the percent of the injected dose per gram wet tissue.

After they were counted, the muscle samples were digested overnight in 1 ml concentrated nitric acid, and the digest was then diluted to 10 ml with deionized water. The calcium and potassium concentrations of these solutions and appropriate blanks were measured by atomic absorption and flame

spectrophotometry, respectively. The results were expressed as micromoles per gram wet tissue.

Plasma enzyme and calcium studies. In a parallel study, plasma creatine phosphokinase (CPK) and calcium were determined in normal rats (n = 18) and at varying times after treatment with DHT (n = 23), induction of ischemic myopathy (n = 39), or both (n = 48). Calcium was determined by atomic absorption spectrophotometry and CPK activity was measured by the method of Rosalki.[46]

Comparison study of ^{99m}Tc EHDP and ^{3}H EHDP. In 12 additional animals (2 control, 4 with ischemic myopathy and 6 with ischemic myopathy following DHT pretreatment), the muscle uptake of both ^{99m}Tc EHDP and ^{3}H EHDP was measured. Ischemic myopathy rats were studied 24 hours after 5-HT injection. Each rat was first injected intravenously with ^{99m}Tc EHDP in the same dose noted above and then 2-3 min later with ^{3}H EHDP (0.5 mg with specific activity 20 μCi/mg in 0.2 ml normal saline). Muscle samples were obtained 2 hours later, weighed, and assayed for ^{99m}Tc as described above. After the ^{99m}Tc had decayed, the muscle samples were digested in 4 ml "NCS" (Amersham/Searle) and the ^{3}H activity was determined by liquid scintillation counting. Results were expressed as percent of the injected dose per gram wet tissue.

Histologic studies. Samples of quadriceps from normal rats and from animals with ischemic myopathy (24 hours after 5-HT) with and without DHT pretreatment were obtained for histologic evaluation. Parallel sections (10 μm thick) of fresh frozen tissue were stained with alizarin red for calcium salts,[33] modified trichrome,[15] nonspecific esterase,[38] NADH-tetrazolium reductase (NADH-TR),[40] alkaline phosphatase,[16] EDTA-reversed ATPase,[13] phosphorylase,[52] acid phosphatase,[9] succinate dehydrogenase[39] and myofibrillar adenosine triphosphatase reactions.[42] Prior to the alizarin red staining some sections were washed either (a) for 2-60 min with 5 mM [ethylenebis(oxyethylenenitrilo)] tetraacetic acid (EGTA) solution, pH 7.0 or 8.0, 20°C, or (b) for 2 min in distilled water.

RESULTS

Tracer distribution and tissue calcium studies. The mean values for muscle ^{99m}Tc EHDP, calcium, and potassium concentrations are shown in Table 1.

Table 1. Muscle Diphosphonate, Calcium, and Potassium Concentrations*

Group	n	^{99m}Tc-Diphosphonate % dose/g x 10^3	Calcium μmoles/g	Potassium μmoles/g
Control	30	13.0 ± 4.4	3.38 ± 0.61	109.4 ± 4.7
DHT only	24	23.6 ± 6.4[a]	3.78 ± 0.73[a]	100.4 ± 5.4[a]
Ischemic Myopathy				
Triceps brachialis	14	12.1 ± 7.2	3.20 ± 0.69	114.8 ± 4.1[a]
Gastrocnemius	14	82.7 ± 85.7[a,d]	4.59 ± 1.96[a,d]	93.0 ± 19.3[a,d]
Quadriceps	14	210.4 ± 85.8[a,d,e]	8.71 ± 4.21[a,d,e]	56.7 ± 26.6[a,d,e]
Ischemic Myopathy + DHT				
Triceps brachialis	16	12.5 ± 4.0[b]	3.79 ± 1.46	110.3 ± 5.2[b,c]
Gastrocnemius	16	99.2 ± 98.1[a,b,d]	4.86 ± 2.22[a,d]	88.0 ± 15.6[a,b,d]
Quadriceps	16	308.2 ± 115.6[a,b,c,d,e]	11.36 ± 5.54[a,b,d,e]	61.6 ± 22.3[a,b,d,e]

*Results presented as mean ± 1 standard deviation

a - c Significantly different by t-test at $p < .05$ compared to control[a], DHT only[b], or to corresponding ischemic myopathy group[c]. The statistical comparisons between the DHT only and ischemic myopathy groups are not shown.

d - e Significantly different by paired t-test at $p < .05$ compared to corresponding triceps brachialis[d] or gastrocnemius[e].

The quadriceps, gastrocnemius, and triceps values were pooled for control animals and for rats treated only with DHT since there were no significant differences between the individual muscles within these two groups. DHT treatment alone resulted in slight increases in muscle ^{99m}Tc EHDP and calcium concentrations and a slight decrease in the potassium concentration.

As we reported previously,[50] ischemic myopathy resulted in markedly increased uptake of ^{99m}Tc EHDP in the quadriceps, accompanied by a decrease of potassium concentration in that muscle. We now find that the calcium concentration is also prominently increased in injured quadriceps. Similar but less marked changes were noted in the gastrocnemii. The triceps brachialis values

were not different from those of control rats except for a slight but statistically significant increase in potassium concentration. This latter change possibly reflects a redistribution of body potassium following a release of potassium from the injured hindlimb muscles.

DHT pretreatment of rats with ischemic myopathy resulted in even greater uptake of ^{99m}Tc EHDP in the quadriceps. The concentration of calcium in the quadriceps of these animals was higher than that in myopathic rats not pretreated with DHT, but the mean values were not significantly different ($p = 0.16$) due to the large variability within each group. The quadriceps potassium concentrations were similar in myopathic rats with and without DHT pretreatment. In the gastrocnemii, similar but less marked differences in EHDP and calcium concentrations were noted between DHT pretreated and nonpretreated ischemic myopathy animals. The triceps values in these animals were not different from those of control rats.

There was a highly significant correlation ($r = 0.778$, $p<0.001$) between ^{99m}Tc EHDP uptake and calcium concentration in the individual muscle samples from all 24 rats (Figure 1).

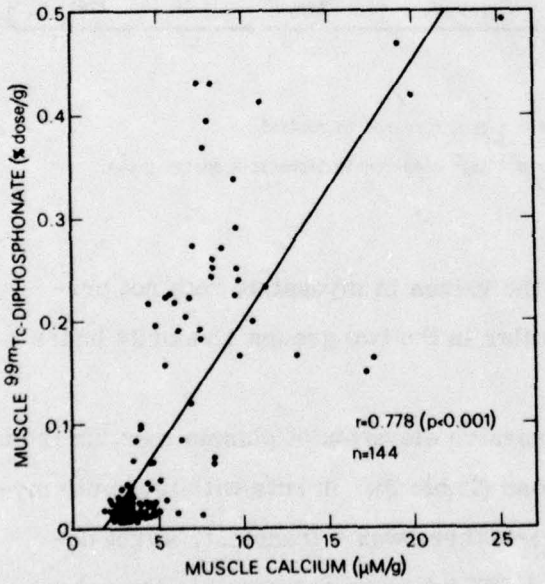


Figure 1.
Relationship between muscle calcium concentration and ^{99m}Tc EHDP uptake. Points represent the values for individual muscle samples. The line shown is the calculated regression line with equation $Y = 0.025X-0.046$.

Plasma enzyme and calcium studies. The plasma CPK concentration was markedly increased after induction of ischemic myopathy (Table 2). Peak values occurred 6 hours after administration of 5-HT. In DHT pretreated rats with ischemic myopathy, plasma CPK values were significantly lower at 3 and 6 hours

Table 2. Plasma Creatine Phosphokinase Concentrations (IU/ml)*

Time after DHT (hr)	Time after 5-HT (hr)	Control	DHT Only	Ischemic Myopathy	Ischemic Myopathy + DHT
-	-	102 ± 42 (18)		254 ± 110[a,b] (5)	
6	-		343 ± 154 (4)		276 ± 145[b] (6)
12	-		164 ± 24[b] (5)		142 ± 64 (6)
24	-		226 ± 93[b] (5)		142 ± 114[a] (6)
27	3			4366 ± 1868[b] (5)	1743 ± 1361[b,c] (6)
30	6			5987 ± 1229[b] (5)	3663 ± 1633[b,c] (5)
36	12			387 ± 137[b] (6)	378 ± 168[b] (5)
48	24		229 ± 175 (9)	193 ± 118[b] (18)	190 ± 55[b] (14)

* Results presented as mean ± 1 standard deviation and (n)

[a] 120 hours after aortic ligation

[b] Statistically significant at $p < 0.05$ by unpaired t-test compared to control

[c] Statistically significant at $p < 0.05$ by unpaired t-test compared to ischemic myopathy group at corresponding time after 5-HT

after 5-HT administration compared to the values in myopathic rats not pretreated with DHT. The values were similar in the two groups 12 and 24 hours after 5-HT injection.

Dihydrotachysterol caused a progressive elevation of plasma calcium from 6 through 48 hours after a single oral dose (Table 3). In rats with ischemic myopathy (with and without DHT pretreatment) there was a transient, slight decrease in plasma calcium 3 hours after 5-HT injection compared to the value

Table 3. Plasma Calcium Concentrations (μmoles/ml)*

Time after DHT (hr)	Time after 5-HT (hr)	Control	DHT Only	Ischemic myopathy	Ischemic myopathy + DHT
-	-	2.58 ± 0.17 (18)		2.62 ± 0.11^{a} (5)	
6	-		3.18 ± 0.19^{b} (4)		2.98 ± 0.08^{b} (6)
12	-		3.40 ± 0.51^{b} (5)		3.01 ± 0.06^{b} (6)
24	-		3.62 ± 0.20^{b} (5)		$3.68 \pm 0.29^{a,b}$ (6)
27	3			2.26 ± 0.20^{b} (5)	$3.36 \pm 0.23^{b,c}$ (6)
30	6			2.44 ± 0.10 (5)	$3.58 \pm 0.51^{b,c}$ (5)
36	12			2.78 ± 0.18^{b} (6)	$3.69 \pm 0.17^{b,c}$ (5)
48	24		3.86 ± 0.62^{b} (9)	2.57 ± 0.32 (18)	$3.67 \pm 0.48^{b,c}$ (14)

* All conditions and footnotes same as in Table 2

just before administration of this drug. This acute change in plasma calcium possibly reflects entry of calcium into injured muscle cells.

Comparison study of ^{99m}Tc EHDP and ^{3}H EHDP. The correlation was excellent ($r = 0.894$, $p < 0.001$) between the muscle uptake of ^{99m}Tc EHDP and ^{3}H EHDP over a wide range of observed values (Figure 2).

Histologic studies. Tissue was examined at 24 hours after 5-HT administration with and without DHT pretreatment (Figures 3-6, Table 4). This experimental myopathy is characterized by the presence of multiple ischemic infarcts of small and large groups of muscle fibers and, in minimally affected areas, of some individual fibers.[35] Three general zones were identified by the alizarin red staining pattern of larger lesions (Figure 3). All skeletal muscle fibers were of approximately normal diameter. The outer zone was narrow, usually about the width of 3-10 fibers, and exhibited marked staining of the fibers for calcium (Figure 5). (Small lesions, less than about 6-20 fibers in

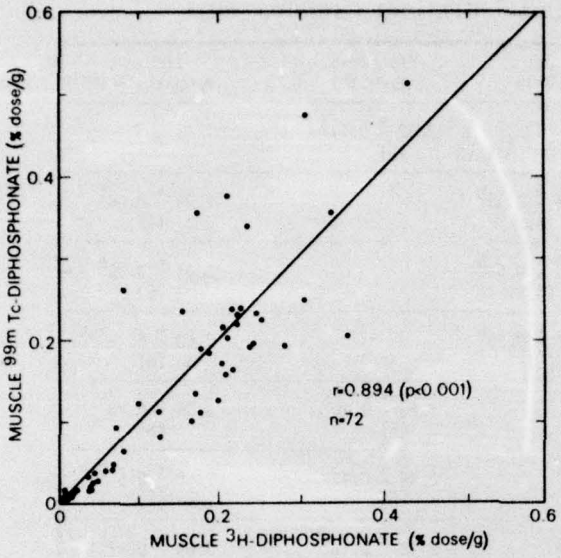


Figure 2.
Relationship between muscle uptake of ^{3}H EHDP and ^{99m}Tc EHDP. Points represent the values for individual muscle samples. The line shown is the calculated regression line with equation Y = 1.010X+0.001.

diameter, exhibited only this staining pattern.) In addition, there was greatly increased staining of the smooth muscle of small arteries and veins within the outer zone. The intermediate zone was usually about 2-4 fibers in width. It had only slightly increased calcium staining of most of the muscle fibers, with a few heavily stained, but no staining in capillaries. The inner zone comprised the bulk of the large lesions (Figure 6). Like the intermediate zone, it had only slightly increased calcium staining of the muscle fibers, but in addition there was marked staining of capillary contents. Those capillaries appeared congested and in some places had exuded their contents into the interstitial region.

Pretreatment with DHT did not alter the appearance of the lesions or the distribution of alizarin red staining. Specifically, there was no evidence of additional extracellular staining to suggest that DHT had produced metastatic calcification. No attempt was made to quantitate the intensity of staining in lesions of animals with and without DHT pretreatment.

Pretreatment of the sections for as little as 2 min with 5 mM EGTA removed all of the alizarin red staining from control muscle and from the histologically normal regions of muscle containing scattered infarcts, except for a

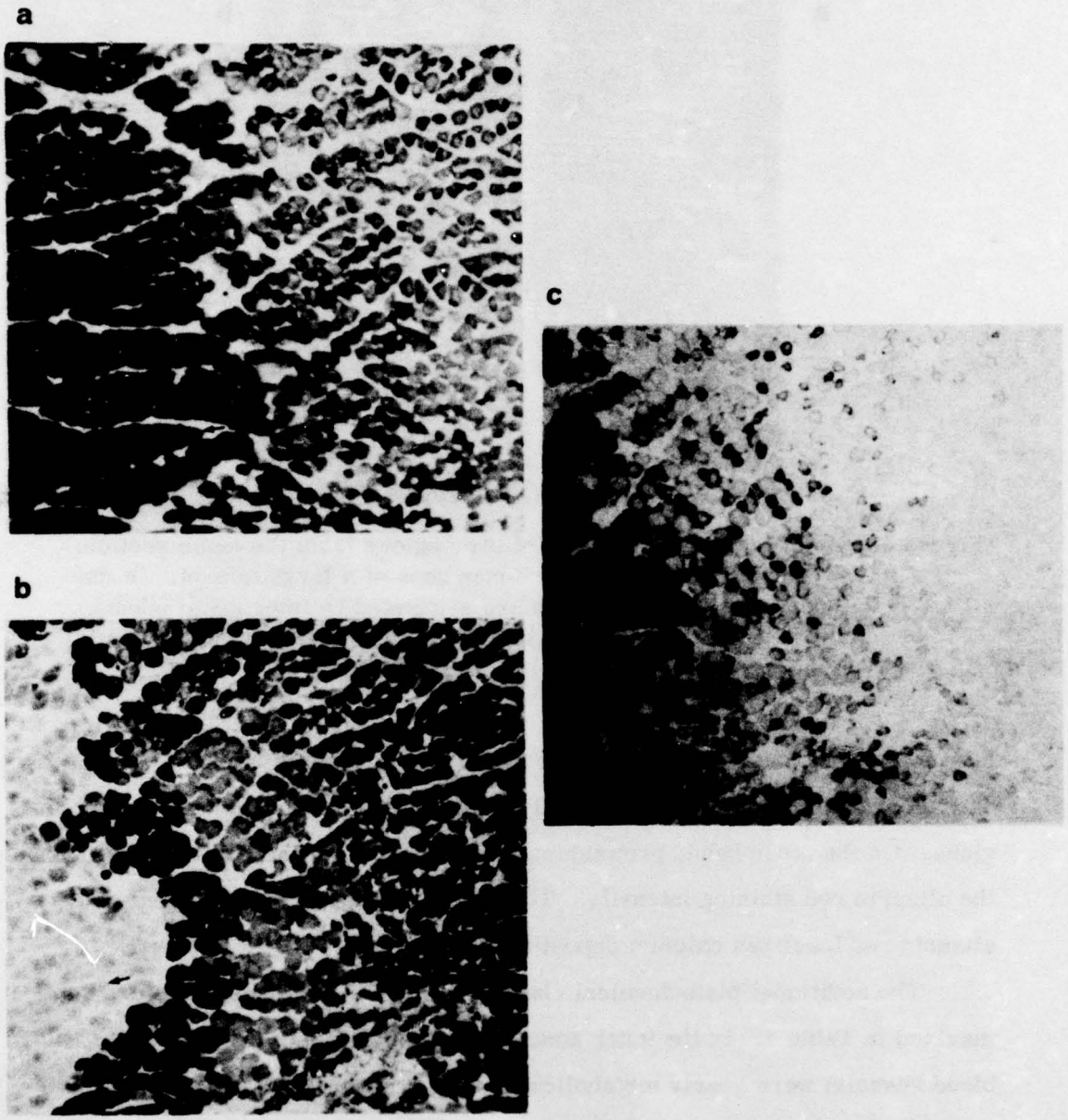


Figure 3. Parallel sections from a large infarct. The normal region is at the left (in that region one fiber is marked with an arrow for comparison of the three sections). a. modified trichrome, b. alizarin red for calcium, c. NADH-TR; all X 48. The calcium stain most clearly demonstrates the three zones within the more darkly stained infarct region (right three-fourths of field) in contrast to the pale normal region (left one-fourth of field). See Table 4 for details.

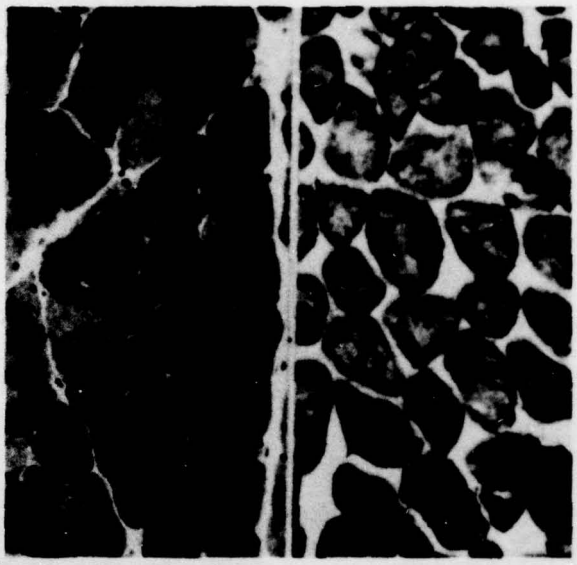


Figure 4. Modified trichrome stain of two regions from the same section: a. normal region, and b. inner zone of a large infarct. In the inner zone, muscle fibers are separated by interstitial edema and have lost the dark staining of their nuclei and intermyofibrillar networks. X 190

faint pink background staining. After 30-60 min incubation in EGTA, the faint background staining diminished equally in both the infarcted and the control regions. On the other hand, prewashing in distilled water for 2 min did not lessen the alizarin red staining intensity. These findings support the contention that alizarin red localizes calcium deposits in tissue.[33]

The additional histochemical characteristics of the three zones are summarized in Table 4. In the inner zone, all tissue elements (muscle fibers and blood vessels) were nearly metabolically dead. In the outer zone, there was a mixture of normal, partially damaged, and phagocytosed muscle fibers. The intermediate zone was transitional, with some metabolically dead fibers and some only partially damaged; however, no phagocytosis was noted. Of particular interest was the phosphorylase reaction, which by its absence sharply delineated all of the abnormal, alizarin red positive fibers, including those

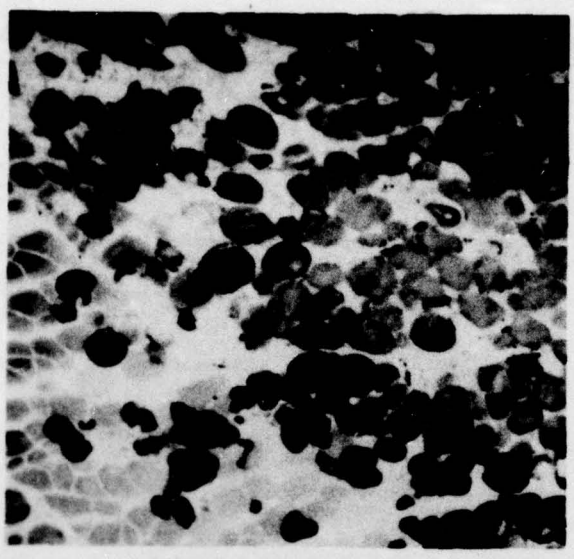


Figure 5. Detail of the border between the normal (lower left) and the infarct regions. Alizarin red for calcium, X 75. All abnormal fibers are stained darker than normal; some in the outer zone are intensely stained throughout and others exhibit punctate staining.

with normal architecture, from the normal alizarin red negative fibers. This reaction thereby appeared to identify the exact extent of the ischemic lesions.

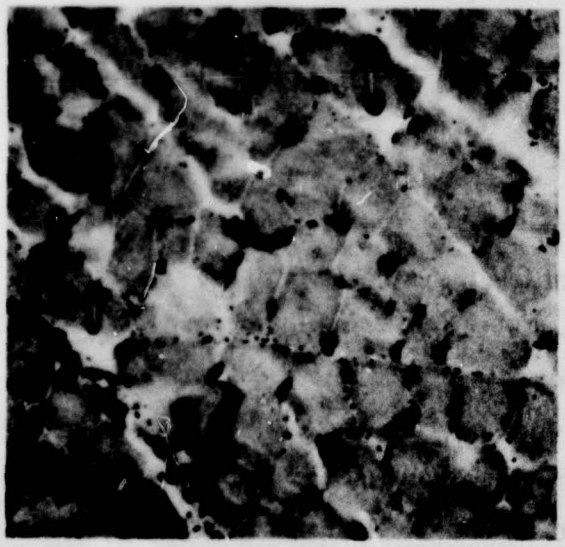


Figure 6.
Detail of the inner zone of an infarct. Alizarin red stain for calcium, X 190. The intensely stained collections are most likely thrombosed capillaries; there is some spillage of their contents into interstitial regions.

Table 4. Summary of Histochemical Findings in Ischemic Myopathic Lesions

Histochemical reaction	Outer zone	Intermediate zone	Inner zone
Modified trichrome	Degenerating fibers and "morphologically intact" fibers. Normal nuclear staining. Interstitial macrophages and neutrophils present with phagocytosis of some damaged fibers.	Red intermyofibrillar network of muscle fibers decreased. Nuclear staining diminished. Rare interstitial exogenous cells.	Red intermyofibrillar network of muscle fibers virtually lost. Nuclear staining virtually lost. Interstitial edema. No interstitial exogenous cells or phagocytosis.
Alizarin red, for calcium	Markedly increased staining of muscle fibers (diffuse or punctate). No capillary congestion or staining. Intense globular staining of smooth muscle of small arteries and veins.	Slightly increased muscle-fiber staining (diffuse and occasionally punctate). No capillary congestion or staining.	Slightly increased muscle-fiber staining (diffuse). Capillaries congested with intensely stained anisotrophic needle-crystals. Some capillary contents spilling into interstitial region.
Phosphorylase	All staining absent. (Directly correlated with increased alizarin red staining.)	All staining absent.	All staining absent.
NADH-TR and succinate-TR	Normal staining in some muscle fibers. Disrupted pattern in some degenerating fibers. In phagocytosed fibers, only macrophages staining. Decreased staining of blood vessel walls.	Decreased staining of muscle fibers. Decreased staining of blood vessel walls.	Markedly decreased staining of muscle fibers and blood vessel walls.
Diaminobenzidine peroxidase	Decreased staining of muscle fibers.	Same as outer zone.	Same as outer zone.
Myofibrillar ATPase	Slightly reduced staining of muscle fibers. Greatly reduced staining of phagocytosed fibers. Medium-sized arteries distended; some thrombosed.	Slightly reduced staining of muscle fibers. Medium-sized arteries distended; some thrombosed.	Slightly reduced staining of muscle fibers. Medium-sized arteries distended; some thrombosed.
Alkaline phosphatase	Staining of proliferating fibroblasts, neutrophils (and ? macrophages). Normal capillary staining.	Absent staining of some capillaries. Rare stained neutrophils (and ? macrophages).	Absent capillary staining. No neutrophils or macrophages present.
Nonspecific esterase	Diffusely decreased or absent staining of muscle fibers. End-plates stained. Absent staining of some capillaries. Macrophages slightly stained.	Absent staining of muscle fibers. End-plates stained. Absent staining of some capillaries.	Absent staining of muscle fibers. End-plates stained. Absent capillary staining.
Acid phosphatase	Staining of macrophages. Slight staining of lumenal contents of occluded arteries. Muscle fiber lysosomes not yet activated.	Slight staining of lumenal contents of occluded arteries. Rare stained macrophages. Muscle fiber lysosomes not yet activated.	Slight staining of lumenal contents of occluded arteries. No macrophages. Muscle fiber lysosomes not yet activated.

DISCUSSION

Our results demonstrate that the accumulation of ^{99m}Tc EHDP in acutely damaged skeletal muscle closely parallels the increase in tissue calcium concentration which occurs after injury. Dihydrotachysterol alone did not cause acute muscle damage. When given to aorta-ligated rats it did not increase the severity of 5-HT induced myopathic injury as reflected by the muscle potassium and plasma CPK concentrations. DHT resulted in sustained hypercalcemia as expected. This was accompanied by, and probably caused, the increased

intracellular deposition of calcium in the injured tissue. This increased tissue calcium probably accounted for the associated further increase in muscle ^{99m}Tc EHDP uptake in DHT pretreated ischemic myopathy rats. These observations suggest that the uptake of ^{99m}Tc EHDP is related to its binding to tissue calcium.

When the ^{99m}Tc EHDP uptake and calcium concentration in injured muscle are compared with those of control tissue, it is apparent that the tracer uptake is increased to a much greater extent than the calcium concentration (Table 1). For example, in the quadriceps of ischemic-myopathy rats, the mean ^{99m}Tc EHDP uptake is increased by a factor of 17.4 compared to the mean triceps value while the calcium concentration increased in the quadriceps by only a factor of 2.7. This observation is not inconsistent with the hypothesis that the ^{99m}Tc EHDP is binding to calcium deposited in the injured tissue. It is likely that the tracer binds to calcium salts deposited as microcrystals, which have been observed by electron microscopy in injured muscle cells,[4,5,7,11,25,48,53] rather than to tissue ionic or protein-bound calcium.[19] Since we have measured total muscle calcium, the relative increase in tissue calcium concentration would underestimate the increase in crystalline calcium deposits.

We have also shown close correspondence between uptake of ^{99m}Tc and ^{3}H labeled EHDP by injured muscle. Our findings are similar to those of Klein et al.[28] who demonstrated good correlation between the uptake of ^{99m}Tc and ^{32}P labeled pyrophosphate in normal and infarcted canine myocardium. These observations suggest that the localization of the ^{99m}Tc stannous phosphate complexes in injured muscle is governed by the properties of the parent compound, i.e., EHDP or pyrophosphate, and is not a unique characteristic of the reduced technetium complex. Thus, ^{3}H EHDP should be a suitable tracer to further evaluate the mechanism of ^{99m}Tc diphosphonate localization in injured tissue by autoradiography, subcellular fractionation, and in vitro binding studies with tissue and tissue components. The greater stability of ^{3}H EHDP and better

autoradiographic resolution achievable with the tritium label would make it preferable to ^{99m}Tc for these types of studies.

The alizarin red method is considered to stain calcium "salts"[33] but not ionic calcium. Therefore the alizarin red staining of the damaged muscle fibers is collateral evidence for deposition of calcium salts in the acute lesions of ischemic myopathy. The sites of calcium salt deposition within the damaged fibers cannot be localized precisely by light microscopy. However, a preliminary ultrastructural histochemical study[41] with pyroantimonate staining of skeletal muscle fibers from rats with experimental muscle damage or humans with spontaneous muscle disease shows pathologic accumulation of calcium in four major subcellular sites. These are (1) sarcoplasmic reticulum (SR), (2) mitochondria, (3) myofibrils (usually in this order of decreasing intensity, although mitochondria occasionally are very heavily laden), and (4) the nucleus (nucleoli and chromatin). There are often collections of calcified SR or mitochondria, which would appear as tiny granules by light microscopy. These cytoplasmic subcellular sites could explain our light microscopic findings and are the same as reported in an earlier nonhistochemical, ultrastructural study of pathologic calcification of skeletal muscle.[5] These same organelles also accumulated calcium after normal rat muscle was soaked in high concentrations of calcium.[41]

Calcification of necrotic myocardial fibers has been described by several investigators.[4,7,11,48,53] Buja et al.[7] have recently shown that the intensity of calcification in experimental canine myocardial infarcts decreases from 2 to 13 days after injury, thus paralleling the evolutionary changes observed on imaging with ^{99m}Tc pyrophosphate. These investigators have also shown histologically that the marked calcium deposition within the muscle fibers occurs chiefly in the reperfused outer regions of the myocardial infarct, and that the uptake of ^{99m}Tc pyrophosphate occurs almost entirely in this region. In contrast, there is virtually no tracer uptake in the nonperfused central zone of the infarct. The localization of calcium and phosphate in mitochondria of abnormal

myocardial muscle fibers has been emphasized by several investigators,[4,7,11,25,48,53] but they have not commented on calcium salt deposition in the myocardial sarcoplasmic reticulum. Crystalline calcium deposits do form in the SR of injured skeletal muscle fibers.[5,41] Since the SR of both tissues normally accumulates calcium avidly, this apparent discrepancy may simply reflect the fact that the SR is less prominent in cardiac muscle compared to skeletal muscle and calcium accumulation in it may have escaped notice.

On the basis of the myocardial studies,[7,32,44] we would predict that ^{99m}Tc EHDP localization in ischemic myopathy lesions should occur only in the zones which are perfused at the time of tracer injection (24 hours after 5-HT administration). In the inner zone, the alizarin red staining material within capillaries suggests microthromboses and, in addition, inflammatory cells are absent. Both of these observations suggest that the inner zone is nonperfused. Thus, in large lesions only the outer zone and part of the intermediate zone would be expected to accumulate ^{99m}Tc EHDP. Reperfusion probably occurs throughout small lesions (6-20 fibers in diameter) and consequently tracer accumulation is likely to be more uniformly increased in them.

The cause of calcium salt accumulation within the organelles of injured cells is not known. The following sequence may represent a likely mechanism. Normally the sarcolemma maintains a large concentration gradient for calcium between the extracellular fluid and the aqueous sarcoplasm.[1,14,21,22,54] It is known that an early manifestation of muscle fiber injury is damage to sarcolemmal integrity. In our model this is demonstrated by loss of muscle potassium and leakage of muscle CPK into plasma.[35,36,50] Other investigators have demonstrated entry of plasma proteins and other macromolecules into minimally injured muscle fibers.[17,27,55] Breaks in the sarcolemmal integrity would allow ingress into injured muscle cells of comparatively large amounts of calcium and subsequently of ^{99m}Tc EHDP which would bind to that calcium. The ability to accumulate calcium is a well documented phenomenon in several subcellular organelles. In frog skeletal muscle, the sarcoplasmic reticulum rapidly

transports calcium into its interior by an active process requiring ATP,[31,34] down to an environmental concentration of 0.1 μM (which is the level necessary for full relaxation of myofibrils).[1,14,21,22,54] Muscle mitochondria take up calcium somewhat more slowly, down to an environmental concentration of about 4 μM, but can accumulate large amounts.[1,30] These calcium-transport functions continue in the organelles after the sarcolemmal membrane is broken and even proceed in cell fractions in vitro. If these organelles remain active for a period of time after sarcolemmal injury with its concomitant increase in the aqueous sarcoplasm calcium concentration, they would be expected to develop the massive calcium salt deposits similar to those demonstrable by electron microscopy when either normal or pathologic muscle is incubated in solutions with a high calcium concentration.[41] Muscle nuclei, and to a lesser extent myofibrils, also accumulate calcium under such incubation conditions,[41] but comparable biochemical studies with isolated nuclei and myofibrils are not available. All of these subcellular accumulations together probably account for the increased muscle fiber calcium we have demonstrated by light microscopy and measurement of total calcium. This increased calcium salt deposition is likely to be responsible for the marked increase in ^{99m}Tc EHDP localization in ischemic myopathic muscle.

The SR and mitochondria of smooth muscle (pig coronary artery) also accumulate calcium avidly.[26] Thus, a similar sarcolemmal leakage mechanism in partially damaged smooth muscle fibers might account for the intense alizarin red staining of the smooth muscle in small arteries and veins within the outer zones of our experimental lesions.

In conclusion, we have demonstrated that calcium salt accumulation occurs in the injured skeletal (and smooth) muscle fibers of rats with ischemic myopathy. The localization of ^{99m}Tc EHDP in injured muscle closely parallels this abnormal calcification process. This phenomenon may be applicable to the clinical and histological identification of abnormal skeletal muscle in various disorders.

REFERENCES

1. Batra, S. The effects of zinc and lanthanum on calcium uptake by mitochondria and fragmented sarcoplasmic reticulum of frog skeletal muscle. J. Cell. Physiol. 82:245-256, 1973.

2. Bonte, F. J., Parkey, R. W., Graham, K. D., Moore, J. and Stokely, E. M. A new method for radionuclide imaging of myocardial infarcts. Radiology 110:473-474, 1974.

3. Bonte, F. J., Parkey, R. W., Graham, K. D., et al. Distribution of several agents useful in imaging myocardial infarcts. J. Nucl. Med. 16:132-135, 1975.

4. Bonucci, E., Derenzini, M. and Marinozzi, V. The organic-inorganic relationship in calcified mitochondria. J. Cell Biol. 59:185-211, 1973.

5. Bonucci, E. and Sadun, R. An electron microscope study on experimental calcification of skeletal muscle. Clin. Orthop. 88:197-217, 1972.

6. Brown, M. W. and Swift, T. R. Radioisotope scanning in inflammatory muscle disease. Neurology 25:347 (Abstract), 1975.

7. Buja, L. M., Parkey, R. W., Dees, J. H., et al. Morphologic correlates of technetium-99m stannous pyrophosphate imaging of acute myocardial infarcts in dogs. Circulation 52:596-607, 1975.

8. Buja, L. M., Tofe, A. J., Parkey, R. W., et al. Technetium phosphate scintigraphy and tissue calcium content in dogs with acute myocardial infarcts. Fed. Proc. 35:230 (Abstract), 1976.

9. Burstone, M. S. Histochemical demonstration of acid phosphatases with naphthol AS phosphates. J. Natl. Cancer Inst. 21:523, 1958.

10. Cook, G. A., Holman, B. L., Westmoreland, N., et al. Localization, detection and sizing of myocardial infarction with technetium-99m-tetracycline and electrophysiologic techniques. Am. J. Cardiol. 33: 131 (Abstract), 1974.

11. D'Agostino, A. N. and Chiga, M. Mitochondrial mineralization in human myocardium. Am. J. Clin. Pathol. 53:820-824, 1970.

12. Dewanjee, M. K. and Prince, E. W. Cellular necrosis model in tissue culture: uptake of ^{99m}Tc-tetracycline and the pertechnetate ion. J. Nucl. Med. 15:577-581, 1974.

13. Drews, G. A. and Engel, W. K. EDTA reversal of muscle fiber activity with the adenosine triphosphatase reaction. Nature 212:1551-1553, 1966.

14. Ebashi, S. and Endo, M. Calcium ion and muscle contraction. Prog. Biophys. Mol. Biol. 18:123-183, 1968.

15. Engel, W. K. and Cunningham, G. G. Rapid examination of muscle tissue -- an improved trichrome method for rapid diagnosis of muscle biopsy fresh-frozen sections. Neurology 13:919-923, 1963.

16. Engel, W. K. and Cunningham, G. G. Alkaline phosphatase-positive abnormal muscle fibers of humans. J. Histochem. Cytochem. 18:55-57, 1970.

17. Fahimi, H. D. and Cotran, R. S. Permeability studies in heat-induced injury of skeletal muscle using lanthanum as fine structural tracer. Am. J. Pathol. 62:143-152, 1971.

18. Fink/Bennett, D., Dworkin, H. J. and Lee, Y.-H. Myocardial imaging of the acute infarct. Radiology 113:449-450, 1974.

19. Francis, M. D., Russell, R. G. G. and Fleisch, H. Disphosphonates inhibit formation of calcium phosphate crystals in vitro and pathological calcification in vivo. Science 165:1264-1266, 1969.

20. Go, R. T., Chie, C. L., Doty, D. B., et al. Radionuclide imaging of experimental myocardial contusion. J. Nucl. Med. 15:1174-1175, 1974.

21. Hadju, S. and Leonard, E. J. A calcium transport system for mammalian cells. Life Sci. 17:1527-1534, 1975.

22. Hasselbach, W. Relaxing factor and the relaxation of muscle. Progr. Biophys. Chem. 14:169-222, 1964.

23. Holman, B. L., Dewanjee, M. K., Idoine, J., Fliegel, C. P., Davis, M. A., Treves, S. and Eldh, P. Detection and localization of experimental myocardial infarction with ^{99m}Tc-tetracycline. J. Nucl. Med. 14:595-599, 1973.

24. Holman, B. L., Lesch, M., Zweiman, F. G., Temte, J., Lown, B. and Gorlin, R. Detection and sizing of acute myocardial infarcts with ^{99m}Tc (Sn) tetracycline. N. Engl. J. Med. 291:159-163, 1974.

25. Jennings, R. B., Moore, C. B., Shen, A. C., et al. Electrolytes of damaged myocardial mitochondria. Proc. Soc. Exp. Biol. Med. 135:515-522, 1970.

26. Jonas, L. and Zelch, U. The subcellular calcium distribution in the smooth muscle cells of the pig coronary artery. Exp. Cell Res. 89: 352-358, 1974.

27. Kent, S. P. Diffusion of plasma proteins into cells: a manifestation of cell injury in rabbit skeletal muscle exposed to lecithinase C. Arch. Pathol. 88:407-412, 1969.

28. Klein, M. S., Coleman, R. E., Ahmed, S. A., et al. ^{99m}Tc(Sn)pyrophosphate scintigraphy: sensitivity, specificity, and mechanisms. Circulation 51-52 (Supplement II):52 (Abstract), 1975.

29. Kula, R. W., Line, B. R., Siegel, B. A., Jones, A. E., Johnson, G. S. and Engel, W. K. ^{99m}Tc-diphosphonate scanning of soft-tissue in neuromuscular diseases. Neurology 26:370 (Abstract), 1976.

30. Lehninger, A. L. Dynamics and mechanics of active ion transport across the mitochondrial membrane. Ann. N. Y. Acad. Sci. 137:700-707, 1966.

31. Makinose, M. Possible functional states of the enzyme of the sarcoplasmic calcium pump. FEBS Lett. 39:140-143, 1973.

32. Martonffy, K., Reimer, K. A., Henkin, R. E., et al. Technetium-99m pyrophosphate concentration in experimental myocardial infarcts. J. Nucl. Med. 16:548 (Abstract), 1975.

33. McGee-Russel, S. M. Histochemical methods for calcium. J. Histochem. Cytochem. 6:22-42, 1958.

34. Meissner, G. ATP and Ca^{2+} binding by the Ca^{2+} pump protein of sarcoplasmic reticulum. Biochim. Biophys. Acta 298:906-926, 1973.

35. Mendell, J. R., Engel, W. K. and Derrer, E. C. Duchenne muscular dystrophy: functional ischemia reproduces its characteristic lesions. Science 172:1143-1145, 1971.

36. Mendell, J. R., Engel, W. K. and Derrer, E. C. Increased plasma enzyme concentrations in rats with functional ischaemia of muscle provide a possible model of Duchenne muscular dystrophy. Nature 239:522-524, 1972.

37. Meroney, W. H., Arney, G. K., Segar, W. E. and Balch, H. H. The acute calcification of traumatized muscle, with particular reference to acute posttraumatic renal insufficiency. J. Clin. Invest. 36:825-832, 1957.

38. Nachlas, M. M. and Seligman, A. M. The histochemical demonstration of esterase. J. Natl. Cancer Inst. 9:415-425, 1949.

39. Nachlas, M. M., Tsou, K.-C., deSouza, E., Cheng, C.-S. and Seligman, A. M. Cytochemical demonstration of succinic dehydrogenase by the use of a new p-nitrophenyl substituted ditetrazole. J. Histochem. Cytochem. 5:420, 1957.

40. Novikoff, A. B., Shin, W.-Y. and Drucker, J. J. Staining results with two tetrazolium salts. J. Biophys. Biochem. Cytol. 9:47-61, 1961.

41. Oberc, M. A. E. and Engel, W. K. Calcium localization in fresh normal and abnormal muscle. J. Cell Biol. 67:312a (Abstract), 1975.

42. Padykula, H. A. and Herman, E. The specificity of the histochemical method of adenosine triphosphatase. J. Histochem. Cytochem. 3:170-195, 1955.

43. Parkey, R. W., Bonte, F. J., Meyer, S. L., Atkins, J. M., Curry, G. L., Stokely, E. M. and Willerson, J. T. A new method for radionuclide imaging of acute myocardial infarction in humans. Circulation 50:540-546, 1974.

44. Poe, N. D., Robinson, G. D., Jr. and Tillisch, J. H. Relation between blood flow and uptake of positive infarct imaging agents. *J. Nucl. Med.* 16:558 (Abstract), 1975.

45. Robinson, G. D., Jr. and Battaglia, D. J. Technetium-99m-tetracycline: preparation, biological dynamics and uptake in damaged and normal skeletal muscle. Int. J. Appl. Radiat. Isot. 26:147-152, 1975.

46. Rosalki, S. B. An improved procedure for serum creatine phosphokinase determination. J. Lab. Clin. Med. 69:696-705, 1967.

47. Shames, D. M., Botvinivk, E., Lappin, H., et al. Quantitation of myocardial infarct size with Tc-99 pyrophosphate and correlation between myocardial CPK depletion and radionuclide uptake. J. Nucl. Med. 16: 569 (Abstract), 1975.

48. Shen, A. C. and Jennings, R. B. Myocardial calcium and magnesium in acute ischemic injury. Am. J. Pathol. 67:417-440, 1972.

49. Shen, A. C. and Jennings, R. B. Kinetics of calcium accumulation in acute myocardial ischemic injury. Am. J. Pathol. 67:441-452, 1972.

50. Siegel, B. A., Engel, W. K. and Derrer, E. C. ^{99m}Tc-diphosphonate uptake in skeletal muscle: a quantitative index of acute damage. Neurology 25:1055-1058, 1975.

51. Stokely, E. M., Lewis, S. E., Buja, L. M., et al. Estimation of acute myocardial infarct size in experimental animals using Tc-99m pyrophosphate (PYP) scintigrams. J. Nucl. Med. 16:573-574 (Abstract), 1975.

52. Takeuchi, T., Higashi, K. and Watanuki, S. Distribution of amylophosphorylase in various tissues of human and mammalian organs. J. Histochem. Cytochem. 3:485-491, 1955.

53. Trump, B. F., Mergner, W. J., Kahng, M. W., et al. Studies on the subcellular pathophysiology of ischemia. Circulation 53 (Supplement I): I-17 - I-26, 1976.

54. Weber, A. Energized calcium transport and relaxing factors. In: Current Topics in Bioenergetics, Sanadi, D. R., editor, pp. 203-254. New York, Academic Press, 1966.

55. Whitaker, J. N. and Engel, W. K. Vascular deposits of immunoglobulin and complement in idiopathic inflammatory myopathy. N. Engl. J. Med. 286:333-338, 1972.

*U.S. GOVERNMENT PRINTING OFFICE: 1976 211-254/695 1-3